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REPORT DOCUMENTATION PAGE

Unclassified	**1b. RESTRICTIVE MARKINGS**
2a. SECURITY CLASSIFICATION AUTHORITY	**3. DISTRIBUTION/AVAILABILITY OF REPORT**
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	Unclassified-available for Unlimited Distribution
4. PERFORMING ORGANIZATION REPORT NUMBER(S) RF 764432/716730	**5. MONITORING ORGANIZATION REPORT NUMBER(S)** AFOSR·TR· 88-0037
6a. NAME OF PERFORMING ORGANIZATION Ohio State University Research Foundation	**6b. OFFICE SYMBOL** *(If applicable)* OSURF	**7a. NAME OF MONITORING ORGANIZATION** AFOSR/NL
6c. ADDRESS *(City, State and ZIP Code)* 1314 Kinnear Road Columbus, Ohio 43212	**7b. ADDRESS** *(City, State and ZIP Code)* Building 410 Bolling AFB DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION United States Air Force	**8b. OFFICE SYMBOL** *(If applicable)* AFOSR/	**9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER** AFOSR-85-0003

DTIC ELECTE FEB 2 0 1988 H

8c. ADDRESS *(City, State and ZIP Code)*
Building 410
Bolling AFB, D.C. 20332

10. SOURCE OF FUNDING NOS.

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT NO.
61102F	2312	A/5	

11. TITLE *(Include Security Classification)* Modulating Transfer RNA Anticodon Modifications and Biologic Responses in Human Cells - Unclassified

12. PERSONAL AUTHOR(S) Ronald Trewyn, Ph.D.

13a. TYPE OF REPORT Final Report	**13b. TIME COVERED** FROM 10/15/85 TO 10/14/87	**14. DATE OF REPORT** *(Yr., Mo., Day)* 1987, December, 15	**15. PAGE COUNT** 10

16. SUPPLEMENTARY NOTATION

17.	COSATI CODES		**18. SUBJECT TERMS** *(Continue on reverse if necessary and identify by block number)*
FIELD	**GROUP**	**SUB. GR.**	human cells, tRNA, queuine, queuosine, hypoxanthine, inosine, differentiation, transformed phenotypes, recombinant RNA, recombinant DNA

19. ABSTRACT *(Continue on reverse if necessary and identify by block number)*

Modulating biologic responses in human cells was shown to be possible by modulating the queuosine modification in the anticodon of specific tRNAs. The molecular basis for guanine analogs including the differentiation of human leukemia cells (reversing expression of the malignant phenotype) was established to be by incorporation into the anticodon in place of queuine, and queuine was able to inhibit the induction of differentiation by these structural analogs. Hypoxanthine analogs were also capable of inducing the differentiation of human leukemia cells, but these compounds were not substrates for the queuosine modification enzyme. In this case AN effect on the inosine modification in the anticodon of other cellular tRNAs appears likely. The purine catabolite hypoxanthine (the natural substrate for the inosine modification) was also to reverse the inhibition of normal human epithelial cell differentiation induced by a phorbol ester tumor promoter.

Characterization of the inosine modification reaction in tRNA required a better unmodified tRNA substrate, so recombinant DNA and RNA technologies were employed to generate

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS ☐	**21. ABSTRACT SECURITY CLASSIFICATION** UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL T. JAN CERVENY, Major, USAF	**22b. TELEPHONE NUMBER** *(Include Area Code)* (202) 767-5021	**22c. OFFICE SYMBOL** NL

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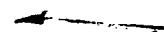
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the required materials. A cloned Bombyx mori alanine tRNA gene was transcribed in vitro and in Escherichia coli and a purified yeast alanine tRNA was subjected to anticodon reconstruction; each of these procedures yielded substrates for the inosine modofication reaction employing extracts of cultural human cells. An antibody raised against inosine was also used to develop a solid phase immunoassay for determining the inosine content in tRNA. Using these various thechniques, the site and nature of the inosine modification reaction was established. Finally, synthetic codons were subcloned into the chloramphenicol acetyl transferase gene for transfection into human cells to correlate changes in the inosine and queuosine modifications to changes in specific gene expression, but these studies have yet to be completed.

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A. RESEARCH OBJECTIVES

This research project was designed to assess the role of tRNA anticodon modifications in regulating gene expression and to determine the potential for manipulating these modifications to modulate biologic responses in human cells. The modification reactions of primary interest were those involved in generating the nucleosides queuosine and inosine exclusively in the first position of the anticodon of specific tRNAs. These are the only two tRNA modifications known to occur by base exchange mechanisms, whereby queuine and hypoxanthine are inserted directly into tRNA macromolecules. Various human cell culture systems were used to determine whether and/or how the dietary factor queuine and the normal purine catabolite hypoxanthine: 1) Inhibit the effects of tumor promoters, 2) block the neoplastic process subsequent to the initiation event, 3) reverse the expression of transformed phenotypes by malignant cells, and 4) induce the maturation of undifferentiated cells. The relationship to specific tRNA structural changes in the anticodon was evaluated.

B. RESEARCH STATUS

A wide variety of agents (e.g., phorbol esters, dimethyl sulfoxide, hypoxanthine, and guanine analogs) will induce the differentiation of leukemia cell in vitro, so human cells of this type were used to examine the potential role of altered tRNA anticodon modifications in this process. A manuscript dealing with our studies related to guanine analog-induced differentiation of human promyelocytic leukemia (HL-60) cells was just published (Kretz et al., Mol. Cell. Biol., 7: 3613-3619, 1987, appended). This study examined the molecular basis for the 6-thioguanine and 8-azaguanine-induced growth inhibition and cell differentiation resulting from treatment of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficient HL-60 cells. 6-Thioguanine and 8-azaguanine are both substrates for the tRNA modification enzyme tRNA-guanine ribosyltransferase, which normally catalyzes the exchange of queuine for guanine exclusively in the first (wobble) position of the anticodon of tRNAs for asparagine, aspartic acid, histidine, and tyrosine. During the early stages of HGPRT-deficient HL-60 cell differentiation induced by 6-thioguanine, there was a transient decrease in the queuine content of tRNA, and changes in the isoacceptor profiles of $tRNA^{His}$ indicated that 6-thioguanine was incorporated into the tRNA in place of queuine. Reversing this structural change in the tRNA anticodon by addition of excess exogenous queuine (at an 800-fold lower concentration than 6-thioguanine) reversed the 6-thioguanine-induced growth inhibition and differentiation. Similar results were obtained when 8-azaguanine replaced 6-thioguanine as the

inducing agent. The data demonstrate that changes in queuine modification of tRNA play a primary role in mediating the differentiation of HGPRT-deficient HL-60 cells induced by guanine analogs.

A related study with HL-60 cells was undertaken with hypoxanthine analogs as well, and a manuscript concerning this work has also been completed (Gibboney and Trewyn, Cancer Res., submitted, appended). While the guanine analogs were incorporated into tRNA by the queuine modification enzyme tRNA-guanine ribosyltransferase, hypoxanthine analogs were not substrates for this enzyme. However, 6-mercaptopurine and 6-ethylmercaptopurine were both able to inhibit the growth and induce functional differentiation of HGPRT-deficient HL-60 cells, with 6-ethylmercaptopurine mediating these responses at significantly lower concentrations than 6-mercaptopurine. In contrast to 6-mercaptopurine, 6-ethylmercaptopurine had similar effects on wild-type (HGPRT-sufficient) HL-60 cells, although it did not induce morphological differentiation of either cell type. At low concentrations, 6-ethylmercapto-purine was not cytotoxic to either HGPRT-sufficient or HGPRT-deficient HL-60 cells, while at high concentrations it was cytotoxic to both. The mechanism by which 6-ethyl-mercaptopurine elicits its effects on HL-60 cells remains to be established, but it appears to be distinct from the mechanism employed by guanine analogs and, perhaps, by 6-mercaptopurine as well. It is possible (and our working hypothesis) that the hypoxanthine analogs are incorporated into the wobble position of tRNAs by the inosine modification enzyme tRNA-hypoxanthine ribosyltransferase, and that this is the mode of action by which they induce the differentiation of HL-60 cells. Based on the bulky nature of the ethylmercapto-moiety in a position normally involved in hydrogen bonding between the codon and anticodon, it could have significant detrimental effects on protein synthesis, and thereby explain the observed differences in 6-mercaptopurine and 6-ethylmercaptopurine. The same bulky moiety could also explain 6-ethylmercaptopurine not being a substrate for HGPRT.

As part of our long-standing collaboration with Dr. Jon Katze at the University of Tennessee Health Sciences Center, a cell line derived from a human malignancy was discovered to totally lack queuosine in the tRNA (Katze et al., Cancer Res., in preparation). Extracts of this colon adenocarcinoma cell line exhibit no detectable tRNA-guanine ribosyltransferase activity, and antibodies developed by Dr. Katze failed to detect any of the large subunit of the enzyme by Western blotting analyses. Since queuine hypomodification of tRNA may be required for neoplastic progression, this could represent the extreme situation where modulation with exogenous queuine would be impossible. It may not be an isolated case however, since these cells

are trisomy 12 and in another malignancy characterized by trisomy 12, CLL, the tRNA may be totally queuosine deficient as well (Emmerich et al., Cancer Res. 45: 4308-4314, 1985).

It was discovered early in the project that the normal substrate for the inosine modification reaction, hypoxanthine, could reverse an effect of the tumor promoter phorbol 12,13-didecanoate (PDD) on normal human epithelial cells in vitro (see Interim Technical Report for the period October 15, 1984 to October 14, 1985). Cultured skin epithelial cells exhibit an upward stratification as they differentiate, and that upward stratification (differentiation) was inhibited by PDD although proliferation of the basal layer was not. Hypoxanthine reversed the effect of PDD and allowed the epithelial cells to differentiate, while having no effect on the untreated control cells. Based on our previous demonstrations that queuine reverses the expression of a PDD-induced transformed phenotype by normal skin fibroblasts (Elliott et al., Cancer Res. 44: 3215-3219, 1984) and that hypoxanthine may induce the differentiation of HL-60 cells by serving as a substrate for the inosine modification of tRNA (Trewyn et al., Proc. Soc. Exp. Biol. Med. 179: 497-503, 1985), we feel that tRNA-hypoxanthine ribosyltransferase may also mediate the effects of hypoxanthine on human epithelial cells. These studies have yet to be completed however.

To monitor changes in the inosine modification of selected tRNAs, a more sensitive, specific assay method was required than was available when the project was initiated. Toward this end, we set out to perfect an immunoassay for determining the inosine content in tRNA, and a manuscript describing this method will soon be published (Yamasaki et al., In: Chromatographic and Other Analytical Methods in Nucleic Acids Modification Research, in press, appended). Reversed-phase high-performance liquid chromatography (HPLC) offers one means for determining the inosine content in hydrolyzed tRNA. Nucleosides in the tRNA hydrolysate are resolved by the HPLC method, and under some circumstances, the inosine content can be established by simple on-line detection based on UV absorbance. However, it is difficult to separate inosine from some of the other modified nucleosides found in unfractionated tRNAs, so an alternative detection method was developed. Inosine-specific, high titer and high affinity antibodies were raised in rabbits against inosine-keyhole limpet hemocyanin conjugate. An enzyme-linked immunosorbant assay (ELISA) was developed, and inosine can be quantitated by competitive inhibition with immobilized antigen. This immunological approach for the quantitation of inosine has proven most effective when the assay is coupled to the reversed-phase HPLC separation of nucleosides in tRNA hydrolysates, but other uses are also likely.

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Modulating the inosine modification in tRNA in cultured human cells required a better understanding of the conditions for hypoxanthine insertion. Therefore, anticodon reconstruction of purified tRNAs (employing recombinant RNA technology) was undertaken to offer a means for characterizing the enzyme(s) responsible for the inosine tRNA modification (Kretz et al., In: Chromatographic and Other Analytical Methods in Nucleic Acids Modification Research, in press, appended). Current knowledge concerning the biosynthesis of modified nucleosides in the anticodon loop of eukaryotic tRNA is limited due to the lack of appropriate substrates for studying these macromolecular modification reactions in vitro and in vivo. Yeast tRNA^{Ala} with the anticodon IGC was reconstructed to contain the anticodon AGC, and this tRNA was used as a substrate for the A_{34} to I_{34} modification catalyzed in vitro by the tRNA-hypoxanthine ribosyltransferase from cultured human HL-60 cells. The tRNA was reconstructed via enzymatic cleavage with Mung bean nuclease to generate the 5' half-molecule and T_1 nuclease to generate the 3' half-molecule. Fragments (lacking the first two nucleotides in the anticodon) were purified by polyacrylamide gel electrophoresis and reannealed prior to ligating a [^{32}P] labelled dinucleotide into the anticodon. The key to utilizing this technology is based on placing the [^{32}P] label adjacent to the nucleoside of interest, so the site specific modification reaction can be monitored. The reconstructed alanine tRNA was then used in an in vitro assay for tRNA-hypoxanthine ribosyltransferase activity in HL-60 cell extracts, and the adenosine to inosine modification was shown to occur.

A broader, generalized summary of the recombinant RNA technology was also published (Grosjean et al., In: Biophosphates and Their Analogues - Synthesis, Structure, Metabolism and Activity, 1987, appended). In addition to covering the A_{34} to I_{34} modification described above (which was the portion funded by this project), this review summarized in vivo studies of the biotransformation of G_{34} to Gm_{34}, Q_{34}, and glycosyl-Q_{34}, of G_{37} to m^1G_{37} and Y_{37}, and of A_{37} to t^6A_{37} and i^6A_{37}. The latter work was all performed in Xenopus laevis oocytes, and while the reason was never elucidated, the A_{34} to I_{34} modification in yeast alanine tRNA was not carried out in this system (Kretz et al., Fed. Proc., 45: 1895, 1986, appended abstract). This modification was carried out with other anticodon reconstructed tRNAs.

An alanine tRNA gene from Bombyx mori which has adenosine in the anticodon wobble position was also used to examine the inosine modification reaction (Trewyn et al., 12th Internatl. Workshop on tRNA, appended abstract; French and Trewyn, Gene, in preparation). By linking this tRNA gene to an SP-6 promoter and subsequently transcribing with the SP-6 polymerase in vitro, an unmodified tRNA^{Ala} precursor was

synthesized. However, although the transcript served as a substrate for the inosine modification in vitro when a crude cell extract from cultured human T-lymphoblasts (CEM cells) was used, it was exceedingly unstable, i.e., greater than 90% of the substrate was degraded during the 60 minute modification assay. Therefore, an alternative procedure was developed, whereby the B. mori alanine tRNA gene was placed under the lac promoter in a bacterial expression vector. Transcription in Escherichia coli was followed by processing of the oversized precursor under appropriate growth conditions, and the tRNA-sized product was at least partially modified in the bacteria. These macromolecular modifications did not occur in the anticodon wobble position however, so after isolating the tRNA from E. coli, it could be used as a substrate for the A_{34} to I_{34} modification in vitro. In fact, this tRNA has proven to be a superb substrate for incorporation of radiolabelled hypoxanthine by the tRNA-hypoxanthine ribosyltransferases from both CEM and HL-60 cells, so characterization of these enzymes is now possible and ongoing.

While the ultimate goal is to understand the significance of changes in the inosine and queuosine modifications in the anticodon wobble position of specific tRNAs during neoplastic progression and cell differentiation, it became obvious that additional model systems for examining specific gene expression would be required if modulation of these modifications was to become feasible in human cells. Toward this end, synthetic oligonucleotides containing five consecutive, identical codons were subcloned into the EcoR1 site of the chloramphenicl acetyl transferase (CAT) gene in bacterial expression vectors. Orientation and reading frame have been confirmed by DNA sequencing for selected leucine [$(CUG)_5$ and $(CUC)_5$] and arginine [$(CGC)_5$ and $(AGA)_5$] codons amorg others. The modified CAT proteins have been detected in bacterial lysates by Western blotting analyses using antibodies specific for either the amino or carboxyl terminus, and the level and rate of expression are being correlated to the content of requisite tRNA isoacceptors in E. coli (French et al., UCLA Symp., submitted, appended abstract; French et al., J. Bacteriol., in preparation). The relative tRNA concentration for the isoacceptors required to read the four codons above is known (an inosine modified tRNA is used to read the arginine CGC codon), so the system is being characterized with E. coli as the more difficult studies with mammalian cells are being undertaken.

Modified CAT gene sequences have also been subcloned into a eukaryotic expression vector and transfected into COS-7, L/A-9, and HL-60 cells; the latter two cell lines both being HGPRT-deficient. With these cells, the influence of the inosine and queuosine modifications in the anticodon of specific tRNAs is being assessed with regard to reading appropriate leucine (I), alanine (I), histidine (Q), and

tyrosine (Q) codons (French et al., UCLA Symp., submitted, appended abstract). The inosine modification is being evaluated in conjunction with the transient over-expression of cloned leucine and alanine tRNA genes as well as with the use of suspected inhibitors of the inosine modification reaction (e.g., 6-mercaptopurine and 6-ethylmercaptopurine). With the queuosine containing tRNAs, the modified (queuosine) and unmodified (guanosine) isoacceptors are being compared to species containing purine analogs (e.g., 6-thioguanosine and 8-azaguanosine). Once the conditions for modulating specific gene expression have been established with the CAT model system, natural processes (e.g., β-interferon and interleukin-2 synthesis) can be examined.

Our studies to date have demonstrated that the dietary factor queuine and the normal purine catabolite hypoxanthine can modulate phenotypic expression by cultured human cells. Major progress was made towards elucidating whether and/or how queuine and hypoxanthine reverse the expression of transformed phenotypes by malignant cells and induce the maturation of undifferentiated cells (Objectives 3 & 4 in Section A). While less progress was made with regard to Objectives 1 & 2, significant effort went into developing the tools and model systems necessary for completing these studies. It was not anticipated when this project was initiated that all aspects would be completed within three years, but it does appear that one can modulate biologic responses in human cells by modulating tRNA anticodon modifications; at least with the queuosine and inosine modifications. These results could have far reaching implications with regard to blocking neoplastic progression subsequent to genotoxin exposure and reversing the expression of transformed phenotypes (inducing differentiation) of already malignant cells.

C. PUBLICATIONS

Articles:

1. Kretz, K.A., Katze, J.R., and Trewyn, R.W. Guanine analog-induced differentiation of human promyelocytic leukemia cells and changes in queuine modification of tRNA. Mol. Cell. Biol. 7: 3613-3619, 1987.

2. Gibboney, D.S., and Trewyn, R.W. Induction of promyelocytic leukemia cell differentiation in vitro by 6-ethylmercaptopurine. Cancer Res., submitted.

3. French, B.T., and Trewyn, R.W. Expression of a Bombyx mori alanine tRNA gene in Escherichia coli. Gene, in preparation.

4. French, B.T., Yamasaki, E.F., and Trewyn, R.W. Differential utilization of synthetic codons in _Escherichia coli_. J. Bacteriol., in preparation.

5. Katze, J.R., Gündüz, U., Elliott, M.S., Houghton, J.A., Houghton, P.J., and Trewyn, R.W. Absence of transfer RNA-guanine ribosyltransferase in cells derived from a human colon adenocarcinoma. Cancer Res., in preparation.

Book Chapters:

1. Grosjean, H., Haumont, E., Droogmans, L., Carbon, P., Fournier, M., de Henau, S., Doi, T., Keith, G., Gangloff, J., Kretz, K., and Trewyn, R. A novel approach to the biosynthesis of modified nucleosides in the anticodon loops of eukaryotic transfer RNAs. In: Biophosphates and Their Analogues - Synthesis, Structure, Metabolism and Activity (K.S. Bruzik and W.J. Stec, eds.), pp. 355-378, Elsevier, Amsterdam, 1987.

2. Yamasaki, E.F., Wani, A.A., and Trewyn, R.W. Solid phase immunoassay for determining the inosine content in transfer RNA. In: Chromatographic and Other Analytical Methods in Nucleic Acids Modification Research (C.W. Gehrke and K.C. Kuo, eds.), Elsevier, Amsterdam, in press.

3. Kretz, K.A., Trewyn, R.W., Keith, G., and Grosjean, H. Site directed replacement of nucleotides in the anticodon loop of tRNA: Application to the study of inosine biosynthesis in yeast tRNA^{Ala}. In: Chromatographic and Other Analytical Methods in Nucleic Acids Modification Research (C.W. Gehrke and K.C. Kuo, eds.), Elsevier, Amsterdam, in press.

Abstracts:

1. Kretz, K.A., Trewyn, R.W., Keith, G., and Grosjean, H. Anticodon reconstruction of inosine-containing transfer RNA species. Fed. Proc. _45_: 1701, 1986.

2. Trewyn, R.W., Kretz, K.A., Roth, A.D., French, B.T., Keith, G., and Grosjean, H. Inosine biosynthesis in the anticodon of transfer RNA. 12th International Workshop on tRNA, Umeå, Sweden, 1987.

3. French, B.T., Yamasaki, E.F., Grever, M.R., Grosjean, H., and Trewyn, R.W. Influence of tRNA content and modification on the translation of synthetic codons _in vivo_. UCLA Symposia - Molecular Biology of RNA, submitted.

D. **PERSONNEL**

Ronald W. Trewyn, Ph.D., Associate Professor of
Physiological Chemistry, Principal Investigator

Bernard T. French, Ph.D., Postdoctoral Research Associate

Edith F. Yamasaki, M.S., Technical Research Associate

Eric D. Utz, B.S., Technical Research Assistant

Keith A. Kretz, Graduate Research Associate
   Thesis: Anticodon Modifications of Transfer RNA and Cell
   Differentiation; Ph.D., 1987.

Andrew D. Roth, Graduate Research Associate

Diane S. Gibboney, Undergraduate Honors Student
   Thesis: Induction of Leukemia Cell Differentiation with
   Hypoxanthine Analogues; B.S., 1987.

E. **INTERACTIONS**

R.W. Trewyn: Symposium on the Transfer-RNA Modification
Queuosine, University of Erlangen-Nürnberg, Erlangen, West
Germany, June 1 to 4, 1985. Presentation: Queuine
Deficient tRNA and Neoplastic Progression.

K.A. Kretz: Reconstruction of the yeast alanine tRNA
anticodon in the laboratory of Professor Henri Grosjean,
Brussels, Belgium, September 4 to November 12, 1985.

R.W. Trewyn: Present a seminar and meet with collaborator
Dr. Jon R. Katze, University of Tennessee Health Sciences
Center, Memphis, Tennessee, October 26 to 28, 1985.
Presentation: tRNA: The Rodney Dangerfield of Molecular
Biology.

K.A. Kretz and R.W. Trewyn: Meeting of the American Society
of Biological Chemists, Washington, D.C., June 8 to 12,
1986. Presentation: Anticodon Reconstruction of Inosine-
Containing Transfer RNA Species.

R.W. Trewyn: Present a seminar at Wright State University,
Dayton, Ohio, May 1, 1987. Presentation: Anticodon
Modification of tRNA and Cell Differentiation.

R.W. Trewyn: 12th International Workshop on tRNA,
University of Umeå, Umeå, Sweden, July 3 to 9, 1987.
Presentation: Inosine Biosynthesis in the Anticodon of
Transfer RNA.

R.W. Trewyn: Symposium on tRNA Modification, University of Umeå, Umeå, Sweden, July 9 to 12, 1987. Presentation: Changes in tRNA Modification and Induction of Leukemia Cell Differentiation.

R.W. Trewyn: Present a seminar at Burroughs Wellcome Co., Research Triangle Park, North Carolina, October 8, 1987. Presentation: Thiopurine Modification of tRNA and Induction of Leukemia Cell Differentiation.

F. APPENDIX

All completed (published, in press, or submitted) articles, book chapters, and abstracts listed in Section C.